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## **Improvement of dolichol-linked oligosaccharide biosynthesis by the squalene synthase inhibitor Zaragozic acid**

Haeuptle, M A ; Welti, M ; Troxler, H ; Huelsmeier, A J ; Imbach, T ; Hennet, T

**Abstract:** The majority of Congenital Disorders of Glycosylation (CDG) are caused by defects of dolichol (Dol)-linked oligosaccharide assembly, which lead to under-occupancy of N-glycosylation sites. Most mutations encountered in CDG are hypomorphic, thus leaving residual activity to the affected biosynthetic enzymes. We hypothesized that increased cellular levels of Dol-linked substrates might compensate for the low biosynthetic activity and thereby improve the output of protein N-glycosylation in CDG. To this end, we have investigated the potential of the squalene synthase inhibitor zaragozic acid to redirect the flow of the poly-isoprene pathway towards Dol by lowering cholesterol biosynthesis. The addition of zaragozic acid to CDG fibroblasts with a Dol-P-Man synthase defect led to the formation of longer Dol-P species and to increased Dol-P-Man levels. This treatment was shown to decrease the pathologic accumulation of incomplete Dol-PP-GlcNAc2Man5 in Dol-P-Man synthase deficient fibroblasts. Zaragozic acid treatment also decreased the amount of truncated protein N-linked oligosaccharides in these CDG fibroblasts. The increased cellular levels of Dol-P-Man and possibly the decreased cholesterol levels in zaragozic acid-treated cells also led to increased availability of the glycosylphosphatidylinositol-anchor as shown by the elevated cell surface expression of the CD59 protein. The present study shows that manipulation of the cellular Dol pool, as achieved by zaragozic acid addition, may represent a valuable approach aimed at improving N-linked glycosylation in CDG cells.

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# IMPROVEMENT OF DOLICHOL-LINKED OLIGOSACCHARIDE BIOSYNTHESIS BY THE SQUALENE SYNTHASE INHIBITOR ZARAGOZIC ACID

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**The majority of Congenital Disorders of Glycosylation (CDG) are caused by defects of dolichol (Dol)-linked oligosaccharide assembly, which lead to under-occupancy of N-glycosylation sites. Most mutations encountered in CDG are hypomorphic, thus leaving residual activity to the affected biosynthetic enzymes. We hypothesized that increased cellular levels of Dol-linked substrates might compensate for the low biosynthetic activity and thereby improve the output of protein N-glycosylation in CDG. To this end, we have investigated the potential of the squalene synthase inhibitor zaragozic acid to redirect the flow of the poly-isoprene pathway towards Dol by lowering cholesterol biosynthesis. The addition of zaragozic acid to CDG fibroblasts with a Dol-P-Man synthase defect led to the formation of longer Dol-P species and to increased Dol-P-Man levels. This treatment was shown to decrease the pathologic accumulation of incomplete Dol-PP-GlcNAc<sub>2</sub>Man<sub>5</sub> in Dol-P-Man synthase deficient fibroblasts. Zaragozic acid treatment also decreased the amount of truncated protein N-linked oligosaccharides in these CDG fibroblasts. The increased cellular levels of Dol-P-Man and possibly the decreased cholesterol levels in zaragozic acid-treated cells also led to increased availability of the glycosylphosphatidylinositol-anchor as shown by the elevated cell surface expression of the CD59 protein. The present study shows that manipulation of the cellular Dol pool, as achieved by zaragozic acid addition, may represent a valuable approach aimed at improving N-linked glycosylation in CDG cells.**

Congenital Disorders of Glycosylation (CDG)<sup>a</sup> are a group of inherited defects of protein glycosylation (1). Mutations in genes encoding either proteins

involved in biosynthesis of lipid-linked oligosaccharide (LLO) required for N-glycosylation (2) or proteins involved in glycan processing (3,4) or transport of N-glycoproteins (5) form the molecular basis of CDG. The majority of CDG encompass disorders affecting the assembly of the LLO precursor dolichol-pyrophosphate (Dol-PP)-GlcNAc<sub>2</sub>Man<sub>9</sub>Glc<sub>3</sub>, which leads to under-occupancy of N-glycosylation sites (6). The stepwise biosynthesis of the LLO precursor begins at the cytosolic side of the endoplasmic reticulum (ER) membrane by transfer of GlcNAc-P to dolichol-P (Dol-P) and completes at the luminal side of the ER membrane. Dol-P does not only serve as carrier of maturing LLO, but also as lipid component of Dol-P-Man and Dol-P-Glc, both donor substrates for luminally acting mannosyl- and glucosyltransferases (7).

The symptoms associated to CDG are principally of neurologic nature, such as psychomotor retardation, ataxia and hypotonia but also include hormonal alterations and coagulopathies (8). The clinical severity of CDG mainly depends on the degree of N-glycosylation site under-occupancy (9), which itself depends on the available pool of complete LLO Dol-PP-GlcNAc<sub>2</sub>Man<sub>9</sub>Glc<sub>3</sub>. To date, only two forms of CDG can be successfully treated by oral carbohydrate supplementation. The glycosylation defects resulting from deficiency of Man-P isomerase (MPI) can be corrected by Man supplementation (10,11), whereas Fuc uptake has been shown to rescue the deficiency of GDP-Fuc transport (12). Considering the involvement of Dol throughout the LLO biosynthetic pathway, we made the hypothesis that increased cellular levels of Dol and Dol-P based substrates may increase the formation of Dol-PP-GlcNAc<sub>2</sub>Man<sub>9</sub>Glc<sub>3</sub> in CDG.

Dol biosynthesis follows the sterol pathway up to the formation of the C<sub>15</sub>-intermediate farnesyl-PP (13) (Fig. 1A). Instead of squalene formation by

head to head assembly of two farnesyl-PP molecules (14), the consecutive condensation of isopentenyl-PP units leads to the diverging synthesis of polyprenyl-PP, a pre-stage of Dol-P. The enzyme squalene synthase catalyzes the first reaction leading exclusively to the formation of sterol compounds, such as cholesterol and steroid hormones (Fig. 1A). Inhibition of squalene synthase, for example by zaragozic acid A (ZGA), leads to the stimulation of prior diverging pathways, and hence to increased formation of Dol and Dol-P (15). ZGA, also known as squalostatin I, was discovered by screening metabolites of filamentous fungi for cholesterol lowering activity (16). Detailed analysis disclosed that ZGA acts as a competitive inhibitor of the squalene synthase by mimicking the farnesyl-PP substrate or the stable intermediate presqualene-PP with its bicyclic, highly acidic core (Fig. 1B). In contrast to statins acting on common steps of both Dol and cholesterol biosynthesis, such as the 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors pravastatin (17), lovastatin (18) or rosuvastatin (19), ZGA does not negatively affect the diverging pathways generating Dol, ubiquinone or prenylated proteins (Fig. 1A). In the present study, we have investigated the ability of the squalene synthase inhibitor to increase Dol biosynthesis and thereby the output of N-glycosylation in CDG fibroblasts.

## EXPERIMENTAL PROCEDURES

**Materials** - ZGA was a gift from Merck & Co., Inc. (Rahway, NJ). The C<sub>80</sub>-polyprenyl-P standards were purchased from Larodan Fine Chemicals (Sweden), and broad range mammalian Dol-P standards were from Sigma-Aldrich (Switzerland). Acetonitrile (Scharlau, Spain), dichloromethane (Sigma-Aldrich) and water (Sigma-Aldrich) were of HPLC grade, other chemicals were of analytical grade.

**Cholesterol analysis** - Human primary skin fibroblasts (4 x 10<sup>7</sup> cells) cultured in DMEM (Sigma-Aldrich) with 10% fetal calf serum (Bioconcept, Switzerland) at 37°C were treated for 72 h with either 100 µM ZGA or DMSO alone as negative control. Cells were harvested by trypsinization, washed in PBS and centrifuged at 1000 x g for 5 min. To the cell pellet 1 ml of 4 % (w/v) KOH in 90 % ethanol was added. After vortexing, the solution was incubated for 10 min in a ultrasonic bath and transferred to a glass tube.

After addition of 80 µl of internal standard (0.1 mg/ml epicoprostanol (Sigma-Aldrich) in pyridine), the solution was saponified for 60 min at 60°C, mixed with 1 ml of water and extracted three times with 2 ml heptane. The pooled heptane extracts were dried under nitrogen and derivatized with 75 µL BSTFA (Machery-Nagel, Oensingen, Switzerland) in 75 µl pyridine at 60°C for 60 min. For GC/MS analysis, the derivative mixture was diluted 5-fold with heptane and 1 µl was injected (injector temperature: 280°C; splitless injection). A Restek RTX-1MS (BGB Analytik AG, Böckten, Switzerland) column (15 m, ID = 0.25 mm) was used for chromatographic separation of the sterols. The carrier gas was helium at a constant flow of 1.5 mL/min. After a dwell time of 3 min at 90°C, the oven temperature was raised to 200°C at 20°C/min, then to 260°C at 1.5°C/min, and finally held at 260°C for 10 min. Mass spectrometry was performed on a Finnigan PolarisQ ion trap mass spectrometer. Mass spectra were acquired in the mass range of m/z = 50 - 550.

**Dol-P analysis** - Fibroblasts (4 x 10<sup>7</sup> cells) were harvested and centrifuged at 1000 x g for 5 min. Cell pellets were dissolved in 12 ml of water/methanol (1:1). Prior to alkaline hydrolysis (20), 15 µg of C<sub>80</sub>-polyprenyl-P standards were added. The extracted Dol and Dol-P were purified on a C<sub>18</sub> Sep Pak column (Waters, USA) and subsequently separated on a Silica Sep Pak column (Waters) (21). The Dol-P were dimethylated using diazomethane generated in an Aldrich diazomethane generator system (Sigma-Aldrich) according to the manufacturer's instructions. The resulting Dol-P-Me<sub>2</sub> were selectively demethylated by overnight incubation in *tert*-butylamine (Sigma-Aldrich) at 70°C (22). Monomethylated Dol-P samples were dissolved in a saturated solution of 9-anthryldiazomethane (Sigma-Aldrich) in diethylether and incubated for 6 h on ice in the dark. Anthracene labeled Dol-P were separated from non-reacted labeling agent by organic extraction (22). The purified products were dissolved in acetonitrile/dichloromethane (3:2) and subjected to HPLC on an Inertsil ODS-3 column (5 µm, 4.6 x 250 mm; GL Sciences Inc., Japan) equipped with a precolumn. Isocratic elution in acetonitrile/dichloromethane (3:2) and 0.01% diethylamine (Sigma-Aldrich) was performed at a flow rate of 1 ml/min (22). Fluorescent labeled Dol-P were detected by excitation at 365 nm and emission at 412 nm. Defined amounts of internal

standard C<sub>80</sub>-polyprenyl-P were used for quantification.

**Determination of Dol-P-Man** - Approximately 4 x 10<sup>7</sup> fibroblasts were grown for 72 h in DMEM containing low Glc (5 mM; Sigma-Aldrich), supplemented with 2% fetal calf serum and 100 µM ZGA or DMSO as negative control. Low Glc medium was utilized to achieve improved Man incorporation (23). Cellular Dol-P-Man was metabolically labeled, extracted and purified according to the protocol of Körner and co-workers (23). Briefly, the fibroblasts were labeled by incubation in DMEM containing 0.5 mM Glc and 125 µCi [<sup>3</sup>H]-Man (Hartmann Analytic, Germany) for 30 min. Dol-P-Man and short LLO were extracted once with chloroform/methanol (2:1) and twice with chloroform/methanol (3:2). The combined organic phases were dried and washed. Thin-layer chromatography on Silica gel 60 plates was performed in chloroform/methanol/water (65:25:4). The plates were analyzed by radiography after signal enhancement with an EN<sup>3</sup>HANCE spray (PerkinElmer, USA) and the area containing Dol-P-Man were scraped and counted in a TRI-CARB 2900 TR liquid scintillation analyzer (Packard, USA).

**CD59 flow cytometry** - Fibroblasts (2 x 10<sup>5</sup> cells) were harvested by trypsinization, washed once in PBS containing 2% fetal calf serum and incubated with a FITC conjugated mouse anti-human CD59 antibody (BD Pharmingen, USA) diluted 1:100 in PBS containing 2% fetal calf serum for 20 min on ice (24). Fibroblasts were washed and analyzed on a BD FACSCanto II flow cytometer (BD Biosciences, USA) equipped with BD FACSDiva software (BD Biosciences).

**LLO and N-linked oligosaccharide (NLO) analysis** - The LLO profiles of 1.5 x 10<sup>7</sup> fibroblasts treated for 72 h with either 100 µM ZGA or DMSO were analyzed as described (25). The cells were starved for 45 min in fetal calf serum- and Glc-free DMEM (Invitrogen) and metabolically labeled for 60 min by addition of 150 µCi [<sup>3</sup>H]-Man. LLO were extracted from cell pellets and oligosaccharides were released by mild acid hydrolysis in 0.1 N HCl. Glycoproteins recovered from the LLO extraction were denatured and NLO were released by overnight incubation with PNGaseF endoglycosidase (New England BioLabs, USA) (26). Oligosaccharides were purified by ion-exchange chromatography on AG1-X2 and AG50W-X8 resins (Bio-Rad, USA) and by

hydrophobic chromatography on Supelclean ENVI-Carb 120/400 beads (Supelco, USA) and C<sub>18</sub> Sep Pak columns and subjected to HPLC analysis.

**Statistics** - Results were expressed as mean ± SEM. The one-way ANOVA test with Bonferroni's multiple comparison post-test was applied to confirm differences between groups. Significance was accepted for p<0.05.

## RESULTS

The toxicity of ZGA was first determined by incubating human primary skin fibroblasts for ten days with increasing concentrations from 10 to 500 µM. ZGA was tolerated by fibroblasts up to a concentration of 125 µM. Above that concentration, the rate of cell proliferation slowed down and cell morphology was altered (data not shown). The addition of 100 µM ZGA to healthy control and CDG fibroblasts led to a moderate decrease of cellular cholesterol levels by 15% and 30%, respectively (Fig. 2).

The effect of ZGA on glycosylation was determined by measuring cellular levels of Dol-P, Dol-P-Man, a glycosylphosphatidylinositol (GPI)-anchored model protein, LLO and NLO. The impact of ZGA on Dol-P levels was first addressed on healthy control fibroblasts. After labeling with the fluorochrome 9-anthryldiazomethane, Dol-P levels were quantitated after HPLC separation (21). The resulting fluorescent HPLC profiles of untreated cells (Fig. 3A) and of cells treated with 100 µM ZGA for 72 h (Fig. 3B) were compared. The pattern of Dol-P species changed upon ZGA treatment, where the amount of longer C<sub>100</sub>- and C<sub>105</sub>-Dol-P increased in ZGA treated fibroblasts (Fig. 3C).

To address the effect of ZGA on various parameters of LLO biosynthesis in CDG, we chose fibroblasts with a deficiency of Dol-P-Man synthase-1 (DPM1) (24), which present low Dol-P-Man levels, an accumulation of the LLO Dol-PP-GlcNAc<sub>2</sub>Man<sub>5</sub> and the corresponding GlcNAc<sub>2</sub>Man<sub>5</sub> glycan structure on glycoproteins. Dol-P-Man production was measured after labeling cells with [<sup>3</sup>H]-Man. The [<sup>3</sup>H]-Dol-P-Man pool determined in DPM1-deficient fibroblasts reached 65% of the levels measured in healthy control cells (Fig. 4). When DPM1-deficient fibroblasts were treated with 100 µM ZGA for 72 h, the [<sup>3</sup>H]-Dol-P-Man levels increased by reaching 120% of normal values. Similarly, when healthy control fibroblasts

were incubated with 100  $\mu$ M ZGA, the levels of [ $^3$ H]-Dol-P-Man increased by 150% (Fig. 4).

DPM1 deficiency leads to reduced cell surface expression of GPI-anchored proteins, since Dol-P-Man is required for the assembly of the GPI anchor (27). Accordingly, low levels of the GPI-anchored protein CD59 have been detected on the cell surface of DPM1-deficient fibroblasts (24). The addition of ZGA led to increased expression of CD59 in healthy control (Fig. 5A) and DPM1-deficient fibroblasts (Fig. 5B), as monitored by flow cytometry. When DPM1-deficient fibroblasts were treated with 100  $\mu$ M ZGA for 72 h, the cell surface levels of CD59 returned to the levels observed in untreated control fibroblasts (Fig. 5C). A time course experiment showed that the ZGA effect was maximal by 72 h of treatment, whereas longer periods did not increase CD59 expression further (Fig. 5C). The effect on CD59 expression was also maximal when applying ZGA at 100  $\mu$ M, but changes were already visible with lower ZGA concentrations (Fig. 5D).

The biosynthesis of the LLO Dol-PP-GlcNAc<sub>2</sub>Man<sub>9</sub>Glc<sub>3</sub> requires Dol as carrier of the growing oligosaccharide and as donor substrate for Dol-P-Man and Dol-P-Glc (7). In normal cells, only the complete LLO Dol-PP-GlcNAc<sub>2</sub>Man<sub>9</sub>Glc<sub>3</sub> can be detected (Fig. 6A). DPM1 deficiency leads to the accumulation of the intermediate LLO Dol-PP-GlcNAc<sub>2</sub>Man<sub>5</sub> (Fig. 6C) (24,28). The amount of this incomplete LLO could be reduced by treatment of DPM1-deficient fibroblasts with 100  $\mu$ M ZGA. The ratio of the abnormal Dol-PP-GlcNAc<sub>2</sub>Man<sub>5</sub> peak to the mature Dol-PP-GlcNAc<sub>2</sub>Man<sub>9</sub>Glc<sub>3</sub> peak was decreased from 54% to 17% under ZGA supplementation (Fig. 6D). By comparison, the addition of 100  $\mu$ M ZGA to control fibroblasts did not influence the quality of the LLO profile (Fig. 6B).

After transfer from LLO to proteins, the NLO GlcNAc<sub>2</sub>Man<sub>9</sub>Glc<sub>3</sub> is trimmed by glucosidases and mannosidases in the ER (29). The analysis of NLO after [ $^3$ H]-Man labeling of control fibroblasts showed, as expected, GlcNAc<sub>2</sub>Man<sub>8</sub>, GlcNAc<sub>2</sub>Man<sub>9</sub> and GlcNAc<sub>2</sub>Man<sub>9</sub>Glc<sub>1</sub> as main oligosaccharide structures (Fig. 7A). In DPM1-deficient fibroblasts, several intermediary oligosaccharide structures ranging from GlcNAc<sub>2</sub>Man<sub>4</sub> to GlcNAc<sub>2</sub>Man<sub>7</sub> were found, whereas the normal oligosaccharides GlcNAc<sub>2</sub>Man<sub>8</sub>, GlcNAc<sub>2</sub>Man<sub>9</sub> and GlcNAc<sub>2</sub>Man<sub>9</sub>Glc<sub>1</sub> were under-represented (Fig. 7C). While ZGA treatment did not affect the NLO

profile of control fibroblasts (Fig. 7B), it decreased the occurrence of abnormal N-linked glycans GlcNAc<sub>2</sub>Man<sub>4</sub> to GlcNAc<sub>2</sub>Man<sub>7</sub> and increased the amounts of the normal NLO GlcNAc<sub>2</sub>Man<sub>8</sub>, GlcNAc<sub>2</sub>Man<sub>9</sub> and GlcNAc<sub>2</sub>Man<sub>9</sub>Glc<sub>1</sub> in DPM1-deficient fibroblasts (Fig. 7D). In these cells, the ratio of the pathologic GlcNAc<sub>2</sub>Man<sub>5</sub> peak to the normal GlcNAc<sub>2</sub>Man<sub>9</sub> peak was 422% and this ratio was decreased to 143% after addition of ZGA (Fig. 7D). This normalization of the NLO profile demonstrated the beneficial effect of ZGA treatment on N-glycosylation.

Taken together, the present study showed that treatment of human CDG fibroblasts with the squalene synthase inhibitor ZGA stimulated Dol-P biosynthesis and thereby improved the level of N-glycosylation in conditions of limited substrate availability as encountered in DPM1-deficiency. In addition, the supplementation with ZGA resulted in the normalization of the expression of the GPI-anchored protein CD59 on DPM1-deficient fibroblasts.

## DISCUSSION

Successful treatment of biosynthetic CDG is restricted so far to oral administration of Man to Man-P isomerase deficient (MPI-CDG) patients (10,11). The applied Man could be phosphorylated by hexokinase and allows functional bypassing of the defective isomerization of fructose-6-P to Man-6-P. In the group of Golgi-associated CDG, a deficiency of the Golgi GDP-Fuc transporter (SLC35C1-CDG or leukocyte adhesion deficiency type II) (30) could also be treated by simple supplementation of a monosaccharide. Oral Fuc administration induced the expression of fucosylated glycoproteins and within short terms, the clinical symptoms of a SLC35C1-CDG patient could be relieved (12). Moreover, a few elementary attempts have been conducted to treat phosphomannomutase-2 deficiency (PMM2-CDG), which forms by far the largest CDG subtype (31). Mutations in the *PMM2* gene lead to disrupted conversion of Man-6-P to Man-1-P, which acts as precursor of GDP-Man (32). However, Man-1-P is neither able to diffuse through biological membranes nor does a transport system exist. Thus, it needs chemical modification prior to direct administration to overcome this obstacle (33-35). Membrane permeable Man-1-P analogues were successful in restoring LLO biosynthesis in CDG patient cells, but such prodrugs were toxic, very

unstable and had to be applied in high concentrations (33).

Shang and Lehrman discovered a metformin-stimulated Man specific transport activity in human fibroblasts (36). The resulting increased Man uptake was shown to be able to correct artificially induced defects in LLO biosynthesis and protein N-glycosylation in control and PMM2-CDG fibroblasts. However, the observation that the clinical phenotype of PMM2 deficiency could not be treated with dietary Man, which was actually effective in cellular models (31), renders a therapeutic administration of metformin questionable.

Our approach to increase Dol-P biosynthesis by inhibition of the squalene synthase offers a therapeutic perspective for inherited biosynthetic N-glycosylation defects featuring residual enzymatic activity. Conveniently, ZGA is well tolerated in animal experiments (37,38) compared to classical statins, which interfere with various isoprenoid-based biosynthesis pathways and are associated to adverse effects (39). We could show that ZGA decreased cellular cholesterol levels, although only by 15-30%, indicating that membrane properties were likely unaltered due to this treatment. However, we cannot exclude an effect of the lowered cholesterol levels on the formation of glycolipid rafts, in which GPI-anchored proteins partition. Accordingly, the increased expression of the GPI-anchored protein CD59 observed in ZGA-treated cells could be caused by increased Dol-P availability and altered raft distribution. By contrast, the positive effect of ZGA supplementation on Dol-P-Man levels and LLO patterns in DPM1-CDG fibroblasts are unlikely to be related to decreased cholesterol biosynthesis since the ER membrane is low in cholesterol levels (40). Nevertheless, the effects of ZGA on protein modifications, such as farnesylation and geranyl-geranylation, have not been investigated, so that we cannot dismiss an impact of such modifications on the N-glycosylation pathway at this stage.

The present study was based on DPM1-deficient fibroblasts because this form of CDG shows several measurable abnormalities along the biosynthesis of N-glycans and GPI-anchored proteins. By contrast, PMM2-deficiency that represents the largest group of CDG cases (6), does not feature accumulating

LLO intermediates. Consequently, incomplete oligosaccharides on glycoproteins are usually not encountered in PMM2-deficiency. However, the up-regulation of Dol-P dependent substrates by ZGA may be beneficial in normalizing the glycosylation disorders caused by deficiency of glycosyltransferases catalyzing the attachment of the last four Man and three Glc of the LLO precursor (7).

Over the last decade, knockout mouse models for PMM2-, MPI- and DPAGT1-deficiency have been generated (41-43). These works clearly showed that disruption of LLO biosynthesis lead to early embryonic lethality. In this context, it would be interesting to investigate whether the embryonic lethality of the homozygotes could be lagged to a later stage of development by treating pregnant heterozygous mice with ZGA according to Keller (15). In addition, the generation of viable CDG mouse models, for instance by introduction of selected point mutations, would allow detailed analysis of organ specific effects of ZGA in the context of inherited N-glycosylation deficiencies. This seems to be of particular interest, considering that Keller reported an enormous effect of ZGA on Dol and Dol-P pools in rat livers, but not in other organs such as brain, kidney, intestine or testis (15), which was similarly observed in different brain cells (44). Subcutaneously administered ZGA might preferentially be taken up by mammalian livers via a specific hepatic transport mechanism, which was likewise proposed in other studies (37,38). Considering the frequent impairment of liver function in CDG patients (8), the proposed hepatic transport system for ZGA would suggest that liver N-glycosylation may benefit from treatment with the compound. Along this line, it would be of interest to address the effect of ZGA supplementation in hepatic cell lines, since fibroblasts do not represent suitable cell models to study glycoprotein secretion. The generation of hepatic cell lines with inactivated glycosylation genes will represent an opportunity to study the regulation of N-glycosylation by ZGA and similar compounds.

In conclusion, this study opens new perspectives in developing treatments of glycosylation deficiency by showing that manipulation of the dolichol biosynthesis pathway, as shown here by ZGA administration, represents a valid option.

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## FOOTNOTES

- <sup>a</sup> The abbreviations used are: CDG, Congenital Disorders of Glycosylation; Dol, dolichol; GPI, glycosylphosphatidylinositol; HMG-CoA, 3-hydroxy-3-methyl-glutaryl Coenzyme A; LLO, lipid-linked oligosaccharides; NLO, N-linked oligosaccharides; ZGA, zaragozic acid A

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## FIGURE LEGENDS

**Figure 1:** Statins acting on the sterol biosynthesis pathway. **(A)** The biosynthetic formation of sterols, such as cholesterol, starts from acetyl-CoA and leads via the C<sub>15</sub>-intermediate farnesyl-PP to its various endproducts. The involved enzymes are numbered as follows: (1) acetoacetyl-CoA thiolase, (2) HMG-CoA synthase, (3) HMG-CoA reductase, (4) mevalonate kinase, (5) phosphomevalonate kinase, (6) mevalonate-PP decarboxylase, (7) isopentenyl-PP isomerase, (8) farnesyl-PP synthase, (9) squalene synthase, (10) squalene monooxygenase, (11) squalene epoxidase, (12) Dol-P-Man synthase, (13) protein farnesyltransferase, (14) geranylgeranyl-PP synthase, (15) protein geranylgeranyltransferase. At the point of farnesyl-PP, the pathway diverges to the formation of prenylated proteins, ubiquinone or Dol. The polyisoprene Dol-P is incorporated into Dol-P-Man, a key intermediate in protein glycosylation and GPI-anchor formation. While pravastatin, lovastatin and rosuvastatin lower cellular cholesterol levels by inhibiting the pathway at the stage of the HMG-CoA reductase, ZGA achieves this by inhibiting the squalene synthase. The chemical structure of ZGA is depicted in **(B)**.

**Figure 2:** Analysis of cholesterol in healthy control and DPM1-CDG fibroblasts. Steady-state cholesterol levels were determined in fibroblasts by GC/MS after organic extraction. Cholesterol levels relative to the protein amounts of the extracted mock treated cells are shown as white bars and cholesterol levels of ZGA treated fibroblasts are shown as gray bars. Data are represented by mean  $\pm$  SEM of three independent experiments, \*  $p < 0.05$ .

**Figure 3:** Analysis and quantification of Dol-P levels in mock and ZGA treated control fibroblasts. **(A)** Control cells were grown in the presence of DMSO. Cellular Dol-P were labeled with the fluorophore 9-anthryldiazomethane and separated by HPLC. The enlarged section in the fluorescent profile highlights the separation of the particular Dol-P species. The elution times of mammalian Dol-P standards ranging from C<sub>90</sub>- to C<sub>105</sub>-Dol-P are indicated below the section. The internal C<sub>80</sub>-polyprenol-P standard utilized for quantification eluted at a retention time of 33.5 min. **(B)** The separation of Dol-P from ZGA treated control fibroblasts is presented in the enlarged section, and the respective Dol-P species were assigned like in **(A)**. Quantification was accomplished by comparison with the internal standard hexadecaprenyl-P eluting at 34.2 min. **(C)** The Dol-P levels from four independent HPLC runs were calculated and normalized to 10<sup>7</sup> fibroblasts. White bar represent Dol-P originating from mock treated and gray bars Dol-P from ZGA treated cells. Data are represented by mean  $\pm$  SEM of four independent experiments, \*  $p < 0.05$ .

**Figure 4:** Determination of Dol-P-Man levels in mock and ZGA treated fibroblasts. Dol-P-Man was metabolically labeled in control and DPM1-CDG fibroblasts by incorporation of [<sup>3</sup>H]-Man and thereafter extracted. The purified lipids were separated by TLC and, on the basis of radiography, the regions corresponding to [<sup>3</sup>H]-Dol-P-Man were scraped and quantified by liquid scintillation. Normalization of Dol-P-Man levels relative to the protein amounts of the extracted mock treated cells are shown as white bars, while the normalized values of ZGA treated fibroblasts are shown as gray bars. Data are represented by mean  $\pm$  SEM of four independent experiments, \*  $p < 0.05$ .

**Figure 5:** Cell surface expression of GPI-anchored CD59. **(A)** Control fibroblasts were stained with a FITC conjugated  $\alpha$ -human CD59 antibody and analysed by flow cytometry. The fluorescent histogram shows unstained cells as gray solid line, mock treated cells as black solid line and ZGA treated cells as black dotted line. **(B)** CD59 expression on DPM1-CDG patient fibroblasts was monitored by flow cytometry. Experimental set up and specimen allocations were like for control cells in **(A)**. **(C)** Control (white bars) and DPM1-CDG fibroblasts (gray bars) were incubated with 100  $\mu$ M ZGA for varying time periods and the detected CD59 expressions were normalized to CD59 levels of untreated control fibroblasts. **(D)** Different concentrations of ZGA were administered to control (white bars) or DPM1-CDG

(gray bars) patient fibroblasts for 72 hours. As in (C), the measured CD59 expressions were normalized to untreated control cells. Data are represented by mean  $\pm$  SEM of three independent experiments.

**Figure 6:** Analysis of ZGA on LLO profiles. Fibroblasts derived from a healthy control (A and B) or a DPM1-CDG patient (C and D) were treated for 72 hours either with 100  $\mu$ M ZGA (B and D) or DMSO as control (A and C). Thereafter, the cells were metabolically labeled with [ $^3$ H]-Man and the LLO were extracted and hydrolyzed by mild acid treatment. The released oligosaccharides were purified and separated by HPLC. The retention time of standard yeast oligosaccharides ranging from GlcNAc<sub>2</sub>Man<sub>1</sub> (M<sub>1</sub>) to GlcNAc<sub>2</sub>Man<sub>9</sub>Glc<sub>3</sub> (M<sub>9</sub>G<sub>3</sub>) are indicated at the top of the profiles.

**Figure 7:** Protein N-glycosylation in ZGA treated fibroblasts. NLO from control (A and B) and DPM1-CDG patient fibroblasts (C and D) were separated by HPLC. The protein linked oligosaccharides were prepared by PNGaseF endoglycosidase release from metabolically labeled cells previously treated for 72 hours either with 100  $\mu$ M ZGA (B and D) or DMSO as control (A and C). The retention times of GlcNAc<sub>2</sub>Man<sub>1</sub> (M<sub>1</sub>) to GlcNAc<sub>2</sub>Man<sub>9</sub>Glc<sub>3</sub> (M<sub>9</sub>G<sub>3</sub>) are marked at the top of the profiles.

## Figure 1

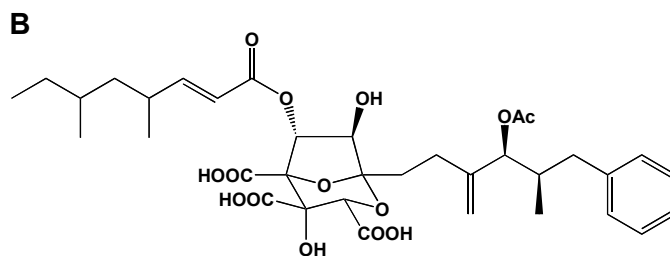
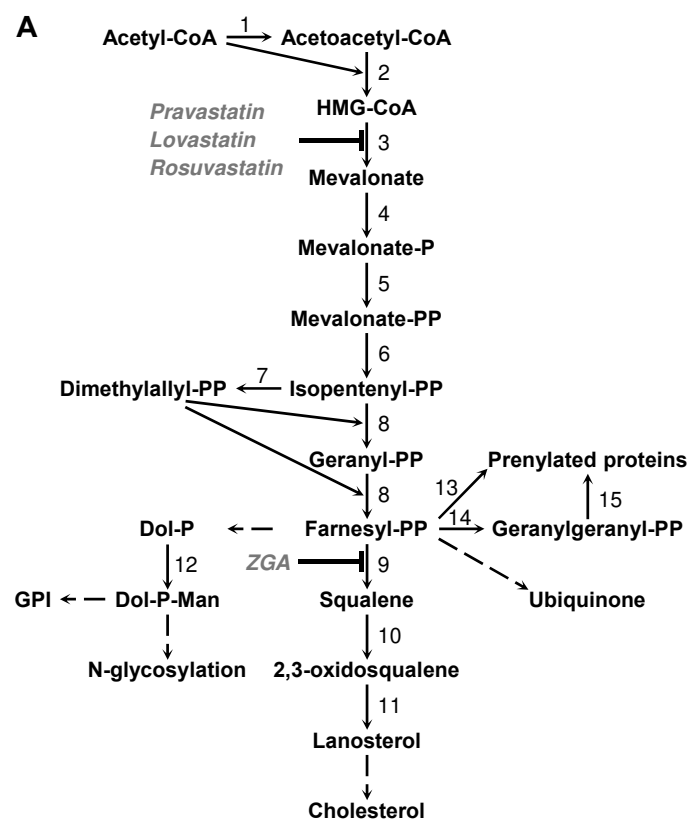


Figure 2

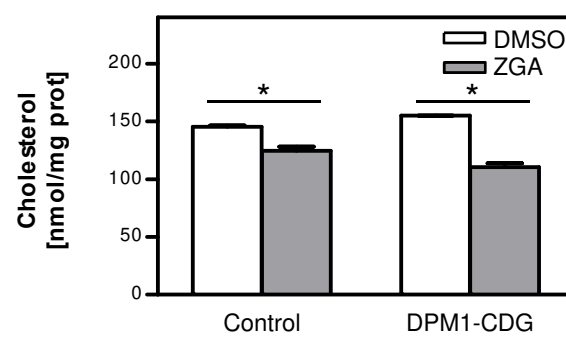


Figure 3

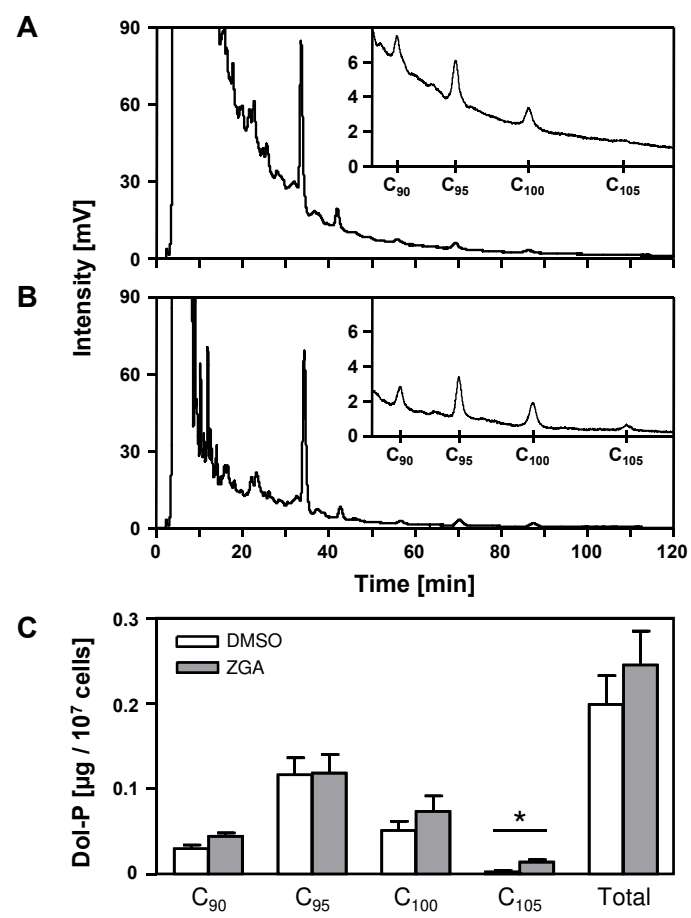


Figure 4

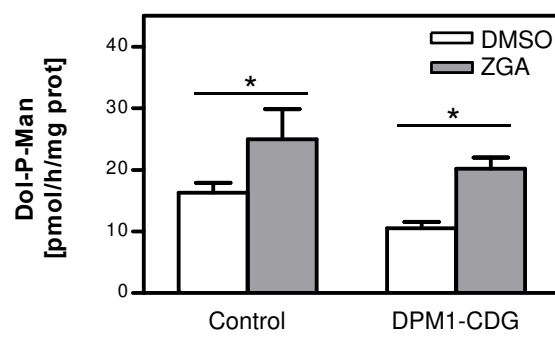


Figure 5

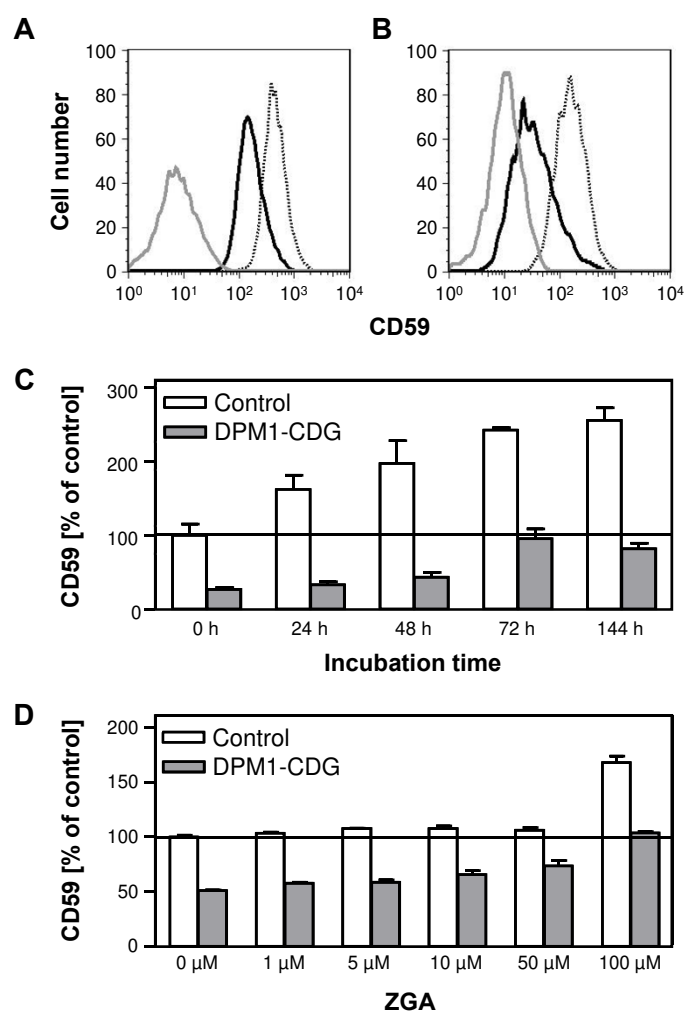


Figure 6

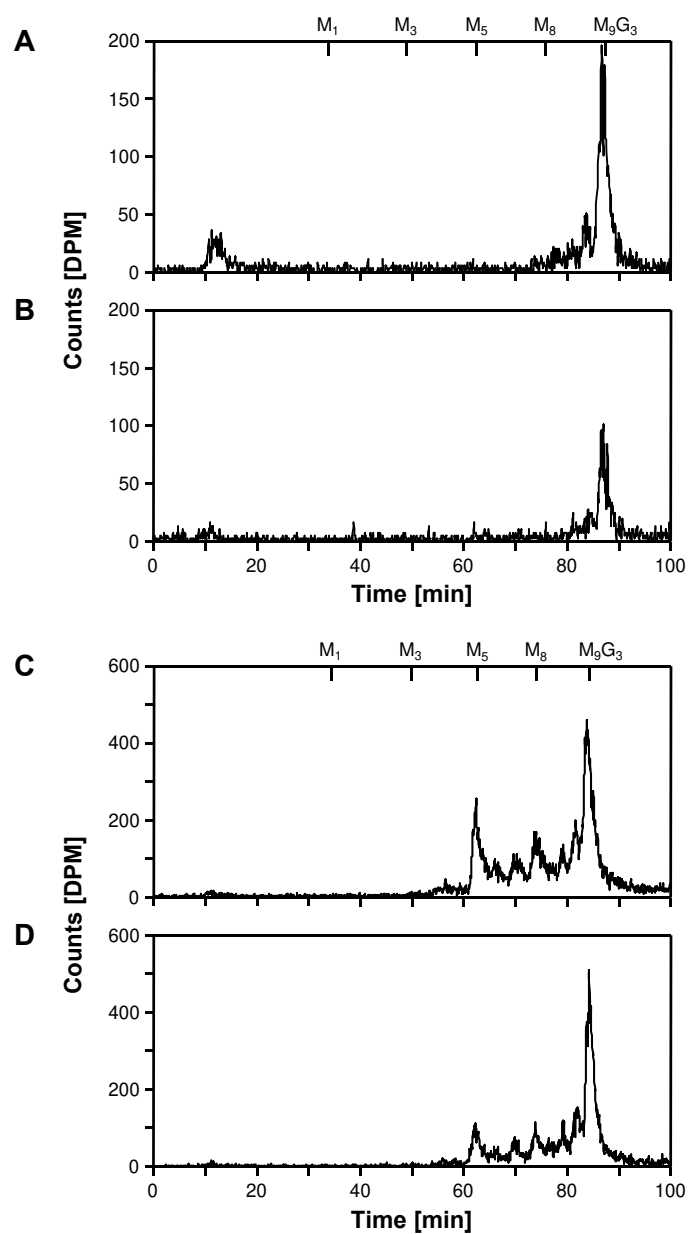




Figure 7

